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Ascorbate oxidase is the potential conductor of a symphony of signaling pathways

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Abbreviations: AM, arbuscular mycorrhizal; AO, ascorbate oxidase; AsA, ascorbic acid; DHA, dehydroascorbic acid; HIF, hypoxia inducible-factor; MDA, monodehydroascorbate; P4H, prolyl-4-hydroxylase; ROS, reactive oxygen species

The functional role of ascorbate oxidase (AO; EC 1.10.3.3) has never been fully explained so far, due to the difficulties in understanding the presence of an enzyme specifically oxidizing ascorbate with no obvious advantage, and the apparent disadvantage of lowering plant stress resistance as a consequence of ascorbate consumption. Here we suggest a complete change of perspective, by proposing an essential role of AO as a modulator of both ascorbate and oxygen content, with relevant implications related to signaling. By affecting the overall redox state, AO is actually involved in redox regulation in the extracellular matrix. In addition, AO can contribute to creating a hypoxic microenvironment, especially relevant in the maintenance of meristem identity and the establishment of mutualistic plant-microbe interactions. We also hypothesize the possible involvement of AO in the activation of a signaling cascade analogous to the mechanism of prolyl hydroxylases/Hypoxia Inducible Factors in animals.

Introduction

The "mysterious enzyme" ascorbate oxidase (AO; EC 1.10.3.3) is a blue copper oxidase displaying several interesting features: (1) it is only found in plants and fungi;[1] (2) it is associated to the cell wall;[2] (3) it catalyzes oxygen reduction to water preferentially using ascorbate (AsA) as the electron donor;[3] (4) its expression and activity are induced by auxin,[4] and light.[5]

All this information, suggesting in part a possible role of AO in signal perception/transduction, has been known for decades, but the function of this remarkable enzyme remained controversial.[6] On the other hand several recent reports, showing that lower AO expression results in higher stress tolerance,[7,8] could lead to the simplistic view that AO activity is dangerous, as it lowers plant defense capability by decreasing the AsA pool. However, this conclusion is clearly misleading, since it does not take into account the tight regulation of AO expression occurring under different developmental and environmental conditions. We have recently reported that a putative AO-coding gene is overexpressed in *Lotus japonicus* during the symbiotic interaction with either nitrogen-fixing bacteria or arbuscular mycorrhizal fungi.[9] Such findings provide new clues to understand the actual function(s) of AO in plants and open the way to novel stimulating hypotheses.

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How Widespread is AO?

After its early discovery in cabbage leaves,[10] AO was found in high amounts mostly in several Cucurbitaceae, and has been considered for a long time an enzyme of limited interest. In recent years, extensive sequencing identified a large number of putative AO genes in many plant species (**Fig. 1**). At least three AO genes are expressed in *Arabidopsis thaliana*, *Lotus japonicus* and *Medicago truncatula*. Protein sequences from Cucurbitaceae (the best characterized from a biochemical point of view) form a distinct clade, which is sister to a large group including two Arabidopsis and several Fabaceae sequences. Other *M. truncatula* and Arabidopsis sequences are more distantly related, together with sequences from *Vitis vinifera* and *Solanum lycopersicum*. Putative AO sequences are also found in lower plants, such as the lycophyte *Selaginella moellendorffii*, the moss *Physcomitrella patens* and the green algae *Chorella* and *Chlamydomonas* (**Fig. 1**), witnessing early appearance of the enzyme in the plant lineage. A functional role for an AO activity in salt adaptation has been suggested in the Chlorophyte *Chaetomorpha linum*.[11] This is compelling evidence that AO is widespread in the plant kingdom, and is likely to have a role in some basic mechanisms of plant adaptation.

Analysis of the promoters of known AO genes using the PLACE software (www.dna.affrc.go.jp/PLACE) suggests light regulation, involvement in nodule formation and regulation by Dof and WRKY transcription factors. Indeed, Esaka and coworkers provided evidence of the apparently specific transcriptional control of an AO gene by a Dof-finger protein they named Ascorbate Oxidase Binding Protein.[12] In addition, some AOs are the potential target of post-transcriptional regulation

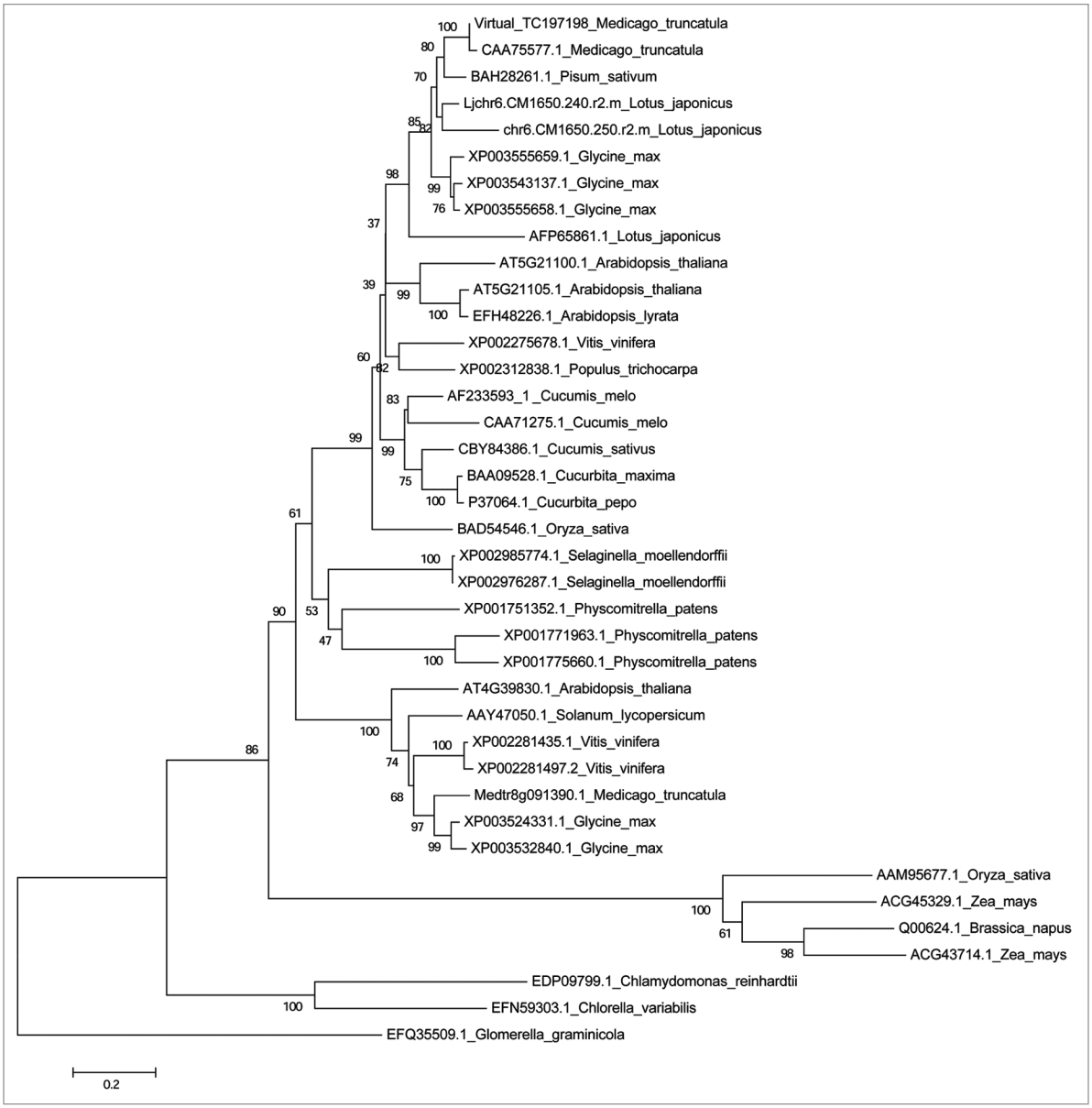

Figure 1. Phylogenetic tree of plant AOs. Phylogenetic relationships between plants based on amino acid deduced sequences for representative ascorbate oxidase (AO) sequences. The sequences were aligned using Muscle (www.ebi.ac.uk/Tools/msa/muscle/) and the alignment tails were trimmed. Phylogenetic tree was constructed with the maximum likelihood method, using the MEGA software, Version 5.0.[30] Bootstrap analyses were conducted on the basis of 1,000 re-samplings of the sequence alignment. A fungal AO sequence has been used as outgroup.

by miRNA.[13,14] Further studies will be necessary to unravel the apparently complex regulation of AO expression.

Redox Regulation

In recent years, the concepts of antioxidant and redox regulation underwent some rethinking.[15] It is now clear that the complex dynamics of oxidants and antioxidants are a practical and efficient way to integrate several cues and trigger both local and systemic responses.[16] By selectively decreasing both oxygen and AsA content in the apoplast, AO apparently works on "both sides" of oxidative stress: on one hand it decreases oxygen content, thus limiting the formation of reactive oxygen species (ROS); on the other hand it oxidizes AsA, which is the main apoplastic antioxidant. The regulation of the apoplastic redox status is key to induce plant responses to both biotic and abiotic stresses.[17] In tomato leaf cells, AO orderly locates in specific apoplastic domains along the plasma membrane, and is particularly abundant in the intercellular spaces (**Fig. 2**). This data is consistent with recent observations, showing that AsA content in cell walls and intercellular spaces is below the detection limit of immunogold labeling techniques,[18] strongly suggesting that AO has an actual role in regulating AsA content. In addition, dehydroascorbic acid (DHA), the oxidized form of AsA, has a role in signaling. The overexpression of cucurbit AO in tobacco plants induced stomata closure, apparently as a result of increased DHA availability.[19] It is noteworthy

that DHA can oxidize protein thiols, and that reversible thiol-disulfide dynamics have been identified as key regulators in signaling.[20]

The "Oxygen Connection"

The AO protein catalyzes a complex reaction, with the safe reduction of molecular oxygen into water, without the release of ROS (hydrogen peroxide, superoxide anion), which result from partial O_2 reduction.[3] This peculiar ability of safely disposing excess oxygen could be of interest under specific conditions.

In green tissues, photosynthetic activity produces large amounts of molecular oxygen, usually released through the stomata. However, in some cases (e.g., low stomatal density, or in CAM plants performing photosynthesis with closed stomata) too much oxygen is potentially harmful, and oxygen quenching is desirable. The hypothesis of a direct involvement of AO under those circumstances is supported by several observations: (a) The induction of AO expression by light; (b) The preferential location of the enzyme in intercellular spaces (**Fig. 2**); (c) data showing that AO activity is induced in CAM plants.[5]

Another interesting application of AO talent in removing oxygen may occur in the specific case of root nodule formation. It is known that oxygen dramatically inhibits nitrogen fixation by rhizobia. We recently reported[9] that *LjAO1* is induced during nodulation in *Lotus japonicus* and is expressed in the peripheral area of the nodule in a possible targeted mechanism controlling oxygen content. Most interestingly, the same gene is also induced during the colonization by an arbuscular mycorrhizal (AM) fungus, suggesting an unexpected more general role in the establishment/functioning of mutualistic plant-microbe interactions.

A third possible aspect of AO action is linked to plant development. High AO activity was first detected by Feldman and coworkers in the root quiescent center (QC), the stem cell niche, ensuring proper functioning of the root meristem.[21] An intriguing relationship is observed between the auxin maximum and the establishment of an oxidizing environment in the QC.[22] As AO activity is auxin-regulated, an involvement of AO in the maintenance of QC cell identity has been hypothesized. Several studies on animal stem cells evidenced the role of hypoxia in maintaining their identity,[23,24] i.e., their ability to proliferate and keep juvenile features.

A recent work,[25] describing the full characterization of the *Lotus japonicus* transcription factor LjMAMI, expressed both in the root meristem and during the AM interaction, also provides a possible explanation for AO involvement in plant-microbe interaction. Arbusculated cells originate from fully differentiated root cortical cells, which have to undergo a complete reprogramming during the colonization by the fungus, going back to a more undifferentiated state. The overexpression of AOs in these tissues[9] could be part of the signaling mechanism related to the change in the developmental program occurring in cortical cells.

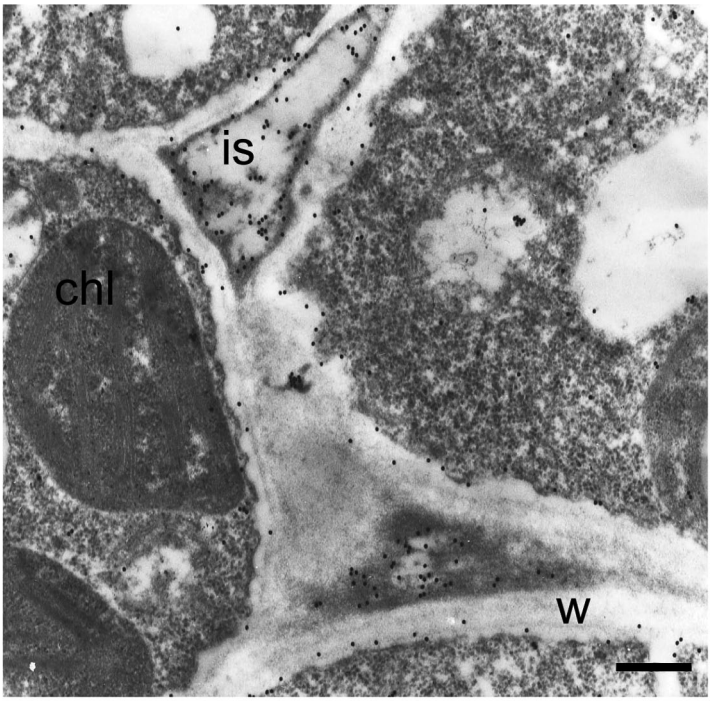

Figure 2. Immunogold localization of AO in young tomato leaves. After treatment with an anti-AO antibody (see ref. 2 for details), labeling (arrows) is mostly present at the peripheral region of the cell wall (w), in close contact with the membrane. No specific labeling is present on chloroplast (chl). Abundant gold granules are present in the intercellular spaces (is). Scale bars: 0.5 μm.

A HIF-Like Signaling System in Plants?

The post-translational hydroxylation of specific proline residues incorporated in polypeptide chains affects protein folding, causing functional changes. The hydroxylation reaction is catalyzed by peptidyl-proly-4-hydroxylase (P4H) using five different co-substrates: peptidyl proline, molecular oxygen, iron (Fe^{2+}), 2-oxoglutarate and AsA. All the co-substrates must be available simultaneously to complete the hydroxylation process. Mature collagen (the most abundant animal protein, required for bone and cartilage integrity) contains several hydroxyproline residues, which are essential to ensure proper functioning of the protein. By the way, the involvement of AsA (vitamin C) in the reaction of collagen hydroxylation is the biochemical basis of the nutritional disorder known as scurvy, which occurs when no adequate dietary AsA supply is assumed by some animals (including humans) unable to synthesize the vitamin.[26] More recently, the hydroxylation reaction has been identified as a key signaling mechanism in mammalian cells. Specific prolyl-hydroxylases have been found, which modify the transcription factor HIF-1α.[27] Successful hydroxylation of two proline residues in the protein targets the transcription factor to ubiquitination, whereas if the two prolines are not hydroxylated, the protein migrates to the nucleus and initiates the transcription of an array of stress-responsive genes.[28] Obviously, the hydroxylation reaction does not take place when one or more co-substrates are not available. It is especially interesting that AO alone can downregulate the

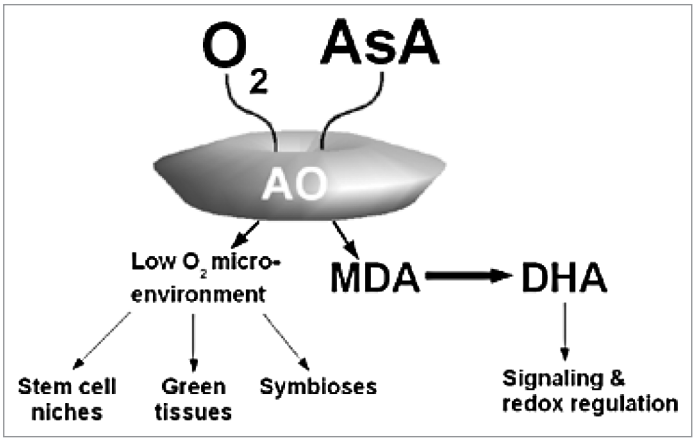

Figure 3. Schematic representation of AO function. AO activity results both in decreasing oxygen level in specific cell compartments or tissues, and in the production of monodehydroascorbate (MDA), which in turn dismutates yielding ascorbate (AsA) and dehydroascorbate (DHA).

availability of two of those substrates, namely AsA and O_2. Only few attempts have been made to identify a hydroxylation-dependent, HIF-like signaling system in plants, with promising results.[29]

Conclusions

Although the identification of the physiological role of AO proved very difficult so far, an increasing amount of data allows us to hypothesize a fundamental regulatory role of the enzyme in several relevant aspects of plant development and survival under stress conditions. A tentative overview of its multiple functions is given in **Figure 3**. Much work will be necessary to complete the picture, and eventually fill the current gaps in our understanding of this potential orchestrator of plant responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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